Differential Expression and Function of Survivin During the Progress of Pterygium

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Purpose. To investigate the expression pattern and function of survivin in the development of pterygium.

Methods. Primary pterygia at quiescent or advanced clinical stage and normal human conjunctival tissues were used in this study. Pterygium epithelial cells (PECs) were cultured in keratinocyte serum-free defined medium and harvested at different growth stages. Tissue sections and cultured cells were detected with survivin, phosphorylated survivin (Thr43), p63, p57, and p21 on protein, and/or mRNA level. Cell Counting Kit (CCK)-8 assay was performed to measure proliferation status of primary cultured PECs. Small interfering (si) RNA specific for survivin was transfected on PECs at subconfluence stage.

Results. Survivin was highly expressed in all pterygium tissues, but not in normal human conjunctiva, at mRNA and protein levels. It was mainly present in the epithelial cytoplasm of pterygium at quiescent stage, while present in the nucleus of pterygium at advanced stage. Phosphorylated survivin was upregulated in pterygium at advanced stage. Pterygium epithelial cells cultured under subconfluence stage showed higher expression of survivin and p63, but lower expression of p57 and p21, compared with PECs reached confluence. Both total and phosphorylated survivin was mainly expressed in the nuclei of PECs under subconfluence, and there was cytoplasmic translocation of survivin when PECs reached confluence. The knockdown of survivin by siRNA inhibited proliferation of PECs, accompanied by downregulation of p63, and upregulation of p57 and p21.

Conclusions. Higher subcellular expression and phosphorylation of survivin may play roles in the development of pterygium. Survivin could be targeted for the treatment of pterygium.

Keywords: survivin, pterygium, cell proliferation

Pterygium is a common ocular surface disease, which can cause irregular corneal astigmatism, corneal stromal scarring, obscuration of the visual axis, and thus leads to vision loss in severe cases.[1,2] Although a variety of surgery treatments are available for the management of pterygium, there are still approximately 2% to 39% of patients will encounter recurrence of the disease.[3–5] Until now there is no effective medication for pterygium treatment. The major reason may be due to the limited understanding of the molecular mechanism regarding the pathogenesis of this disease. A large number of studies have proposed that DNA damage,[6] molecular genetic alterations,[7–9] disruption of apoptosis,[10] cytokines,[11,12] growth factors,[13–16] angiogenic factors,[17,18] extracellular matrix remodeling,[19] immunologic mechanisms,[20,21] and viral infections[22–24] are involved in pathogenesis of pterygium. Yet, a precise molecular mechanism of pterygium pathogenesis remains unclear.

Recent reports have shown that survivin, a member of the inhibitor of apoptosis (IAP) gene family, was expressed in the epithelium of pterygium.[6,25,26] In these studies, survivin was found to be expressed both in the nucleus and in the cytoplasm of the pterygium epithelial cells, however, the specific function of survivin in these cell compartments during pterygium development has not been demonstrated.

Survivin was found highly expressed in fetal tissues and tumors, especially in malignancy, but not present in most of the normal adult tissues.[27] The general function of this protein is related to the inhibition of apoptosis and promotion of cell cycle progression.[28,29] Nuclear survivin has been regarded as prognostic marker of malignant tumor.[30] Downregulation of survivin expression can decrease proliferation and viability of tumor cells in culture and reduce the growth of tumor in mouse models.[31] Therefore, it has been recognized as a specific target in cancer therapy. In addition, some studies have defined the role of survivin in regulating function in normal adult cells, particularly stem cells,[32,33] endothelial cells,[34] and gastrointestinal epithelial cells.[35] Recent studies revealed that survivin was expressed in ocular surface, and it may be involved into Wnt signaling pathway, which could maintain a less differentiated phenotype and high proliferative capacity of human corneal epithelial progenitor cells.[36,37]

Previous studies have revealed that survivin can be localized both inside and outside the cells. The cellular pools of survivin can be nuclear, mitochondrial, and cytoplasmic,[38–40] and the differential localization of survivin has been related with different functions of this protein. Survivin regulates cell division when it is present in the nucleus, while the mitochondria localization is associated with the inhibition of

apoptosis and oncogenic transformation. Moreover, the extracellular pool of survivin is proposed to inhibit apoptosis in cancer cells while increase their proliferation and aggressiveness.[38,41,42]

In this study, we were keen to investigate the cellular localization of survivin in pterygium and the possible function of survivin of pterygium in different clinical stages as well as ex vivo cultured pterygium epithelial cells in different growing stages. The relative signaling pathway molecules of survivin in pterygium epithelial cells was further studied.

Materials and Methods

Patients and Tissues

The Medical Ethics Committee of Xiamen Eye Center (Fujian, China) approved this study. Human tissues were handled according to the Declaration of Helsinki. Written, informed consent was obtained from all participant patients. Forty-two patients with primary pterygia (19 males and 23 females, ages ranged between 39 and 74 years, mean age: 55.5 ± 8.9) were recruited at Xiamen Eye Center. The external eye of each patient was photographed before the pterygium surgery. Pterygia were classified into two stages based on the invasion of the pterygium, the amount of new blood vessels and the transparency of the tissue. Quiescent stage (20 cases) was defined as mild invasion, hyperemia, and moderate transparency, while advanced stage (22 cases) was defined as aggression, dense neovascularization, and opacities of the pterygium tissue. Pterygium tissues were collected immediately after excision and preserved in Keratinocyte serum-free defined medium (KSFM) medium at 4°C for less than 4 hours prior to experiments. Normal conjunctiva tissues were obtained from the eye bank of Xiamen Eye Center.

Reagents and Antibodies

Rabbit polyclonal anti-survivin antibody (ab469) and anti-p63 antibody (ab53039) were purchased from Abcam Biotechnology (Cambridge, UK). The antibodies for phosphorylated survivin (Tr43), p63 (TAp63 and deltaNp63), p57, p21, and lamin B (sc-23758, sc-25268, sc-1040, sc-756, sc-6216) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Beta-actin antibody (A-3854) was from Sigma-Aldrich Corp. (St. Louis, MO, USA). Horse radish peroxidase (HRP)–conjugated goat anti-rabbit IgG antibody (170-6515) was from Bio-Rad (Hercules, CA, USA). Fluorescein isothiocyanate–conjugated swine anti-rabbit IgG antibody (F0205) was from Dako Biotechnology (Glostrup, Denmark). Anti-rabbit Isotype control IgG (08-6199) was from Invitrogen (Carlsbad, CA, USA).

Reverse Transcription System was from Promega (Madison, WI, USA). SYBR Premix Ex Taq (Tli RNaseH Plus) was purchased from TaKaRa (Shiga, Japan). Mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA, USA). Two-step histostaining reagent for rabbit and diaminobenzidine (DAB) were from ZSGB-BIO (Beijing, China). Keratinocyte serum-free defined medium, epidermal growth factor (EGF), and Lipofectamine 2000 were purchased from Invitrogen. BIRC5 siGENOME small interfering (si) RNA (si-suv) was purchased from Dharmacon (Pittsburgh, PA, USA), silencer negative control #1 siRNA (si-ctrl) was from Ambion (Austin, TX, USA). Nucleus and Cytoplasm extract kit was obtained from Beyotime Biotechnology (Haimen, China). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). Enhanced chemiluminescent (ECL) detection system was from Lulong, Inc. (Xiamen, China). Cell Counting Kit (CCK)-8 assay kit was obtained from Dojindo (Tokyo, Japan).

Pterygium Epithelial Cell (PEC) Culture

Pterygium specimens were cut into small pieces 1 to 2 mm in diameter under a stereomicroscope, rinsed with PBS, and placed in 6-well plate with epithelium up. Pterygium epithelial cells with the similar shape climbed out from the edges of pterygium specimens 2 days later. When cell confluence was up to 90%, pterygium specimens were abandoned, and TrypLE-express (an animal origin-free recombinant enzyme) was used to dissociate cells to obtain a single cell suspension. Keratinocyte serum-free defined medium (serum free) was used for cultures at 37°C under 5% CO2 and 95% humidity. Medium was replaced every other day. The second passage of PECs was collected in different growing stages with 70% or 100% confluence for study. Cells with 70% confluence are in exponential growth stage, comparing the cells with 100% confluent in quiescent stage.[37]

RNA Interference

In brief, primary PECs (3.5×10^4 cells per cm^2) were transfected with siRNA specific for survivin (si-suv) and a noncoding sequence siRNA as negative control (si-ctrl) using Lipofectamine 2000 reagent after 24 hours of cell passage and the cell density was approximately 50%. The amount of siRNA is 100 pmol for 6-well format and 5 pmol for 96-well format, respectively. The transfected cells were incubated for 48 hours for RNA isolation, protein extraction, immunostaining or CCK8 assay.

RNA Isolation and Relative Quantitative Realtime PCR

Normal conjunctival and pterygium were incubated with Dispase II (2.5 mg/mL; Roche, Basel, Basel-Stadt, Switzerland) at 4°C for 16 hours. The epithelial sheets were then collected and total RNA was extracted using Trizol (Invitrogen). One microgram of total RNA was used to synthesize the first cDNA strand using Reverse Transcription System of Promega according to the manufacturer's protocol. Primer sequences for survivin (Forward: CAAGGACCACCGCATCTCTAC; Reverse: AGTCTGGCTCGTTCTCAGTGG), p63 (Forward: GAAACCAGA GATGGGCAAGTC; Reverse: TATCTTCATCCGCCTTCCTGT), p57 (Forward: GCGGCGATCAAGAAGCTGT; Reverse: ATCGCCCGACGACTTCTCA), and p21 (Forward: ACCATGTG GACCTGTCACTGTC; Reverse: GGCGTTTGGAGTGGTA GAAATC) were selected from primer 3. Quantitative real-time PCR was performed with a 10-μL reaction volume containing 1 μL template cDNA, 5 μL SYBR green mastermix, 0.4 μL PCR forward primer, 0.4 μL PCR reverse primer, 0.2 μL ROX reference dye (ABI, Foster City, CA, USA), and 3 μL DdH_2O. The reaction was at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and analyzed in StepOne Plus (Applied Biosystems, Foster City, CA, USA).

Immunostaining

Tissue sections were fixed in cold acetone (−20°C) for 10 minutes for immunostaining. Then sections were incubated with 0.2% Triton X-100, washed in PBS, blocked, and incubated with anti-phosphorylated survivin antibody (1:100) at 4°C for 14 hours, after that, the tissues were incubated with swine anti-rabbit IgG (1:50) for 1 hour at room temperature, washed in PBS, counterstained with DAPI and photographed by the confocal laser scanning microscope (Olympus Fluoview

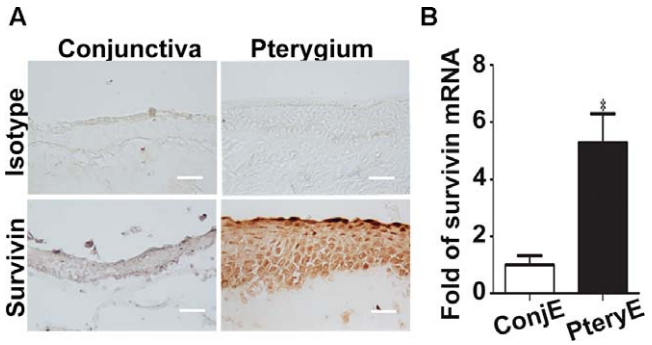

FIGURE 1. The expression of survivin in human conjunctiva and pterygium. (**A**) Representative images of immunohistochemical staining of survivin in human normal conjunctiva and pterygium tissues, staining with isotype IgG as a negative control. *Scale bars*: 100 μm. (**B**) Real-time PCR detection of survivin mRNA expression (relative fold) in human normal conjunctival and in pterygial epithelium (conjE and pteryE). Data were represented as mean ± SEM, $n = 5$; $^*P < 0.05$ versus conjE.

FV1000; Tokyo, Japan). For immunohistochemical staining, sections after fixation were incubated with 0.6% hydrogen peroxide solution for 10 minutes, 0.2% Triton X-100 for 15 minutes, blocking buffer for 60 minutes and anti-survivin antibody (1:400) or anti-p63 antibody (1:200) for 14 hours in proper sequence. Then the sections were further treated with two-step histostaining reagent following the manufacturer's protocol, developed with DAB, and photographed by Nikon Eclipse 50i microscope (Tokyo, Japan).

Western Blotting

Proteins of PECs were extracted with cold RIPA buffer or collected using nucleus and cytoplasm extract kit according to the manufacture's protocol. Twenty micrograms of protein were analyzed under reducing conditions on 12% polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked in PBS buffer (pH 7.4, with 0.05% Tween 20 and 2% BSA) for 30 minutes and incubated with specific primary antibodies: anti-survivin (1:1000), anti-phosphorylated survivin (1:200), anti-p63 (1:400), anti-p57 (1:200), anti-p21 (1:200), and anti-β-actin (1:10000). Then membranes were washed in PBS with 0.05% Tween 20, incubated with peroxidase-conjugated secondary goat anti-rabbit antibody (1:7500) for 1 hour at room temperature, washed, and developed using the ECL detection system.

CCK-8 Assay

Cell Counting Kit (CCK-8/WST-8) was used to measure cell proliferation of PECs according to the manufacturer's protocol. In brief, when cell confluence was up to 90%, TrypLE-express (an animal origin–free recombinant enzyme) was used to dissociate cells to obtain a single cell suspension. Ten thousand pterygium cells were seeded in each well of 96-well plate with 100 μL culture medium. Three repeats were performed in this experiment. Pterygium epithelial cells were transfected with siRNA specific for survivin (si-suv) and a noncoding sequence siRNA as negative control (si-ctrl) using Lipofectamine 2000 reagent after 24 hours of cell passage and the cell density was approximately 50%. Then, 10 μL CCK8 reagent was added into each well of 96-well plate with 100 μL culture medium, and then cells were incubated for 3 hours at 37°C in a 5% CO_2 incubator. Cell proliferation was monitored at a wavelength of 570 nm with a Bio-Tek ELX800 microplate reader (Winooski, VT, USA).

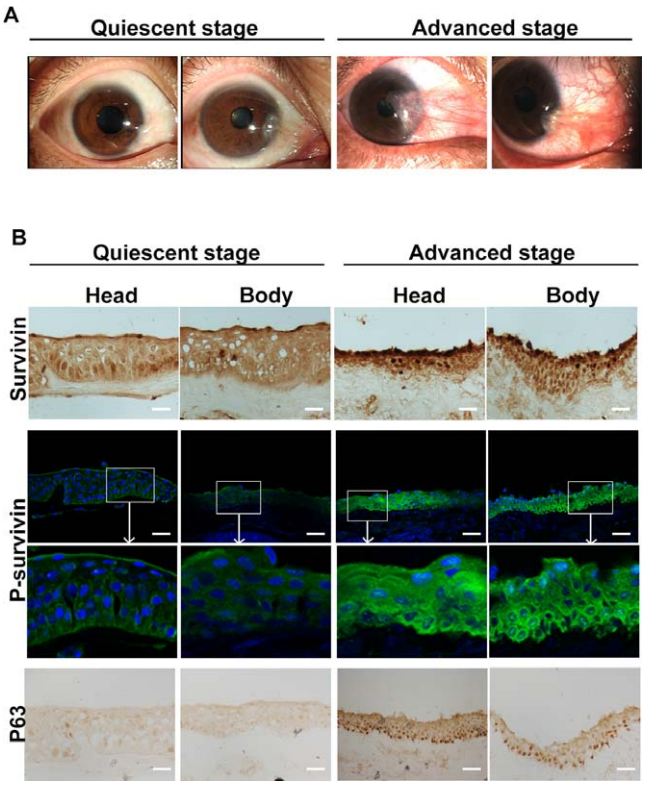

FIGURE 2. The nuclear translocation and phosphorylation of survivin in pterygium tissues at different clinical stage. (**A**) Representative images of the external eye of patients with pterygium at different clinical stages. (**B**) Representative images of immunostaining of survivin, phosphorylated surviving, and p63. *Scale bars*: 50 μm.

Statistical Analysis

Summary data are reported as means ± SEM. The Student's unpaired *t*-test was applied in the analysis of all experimental data. Test results are two-tailed, where P less than 0.05 is considered statistically significant.

RESULTS

Survivin was Expressed in Pterygium

We first detected the gene expression of survivin in normal human conjunctiva and pterygium tissues. Immunohistochemical staining revealed that survivin was highly expressed in all pterygium tissues and located in both nucleus and cytoplasm of epithelial cells, while it was only faintly expressed in the cytoplasm of normal human conjunctival epithelium (Fig. 1A). There was no obvious expression in mesenchymal cells. Meanwhile, real-time PCR data showed that there was a significant increase of survivin mRNA expression in pterygium over normal conjunctival tissue by approximately 5-fold ($P < 0.05$, $n = 5$; Fig. 1B).

The Expression of Survivin in Pterygium of Different Clinical Stages

The external eye of each patient was photographed and representative images were shown in Figure 2A. We then

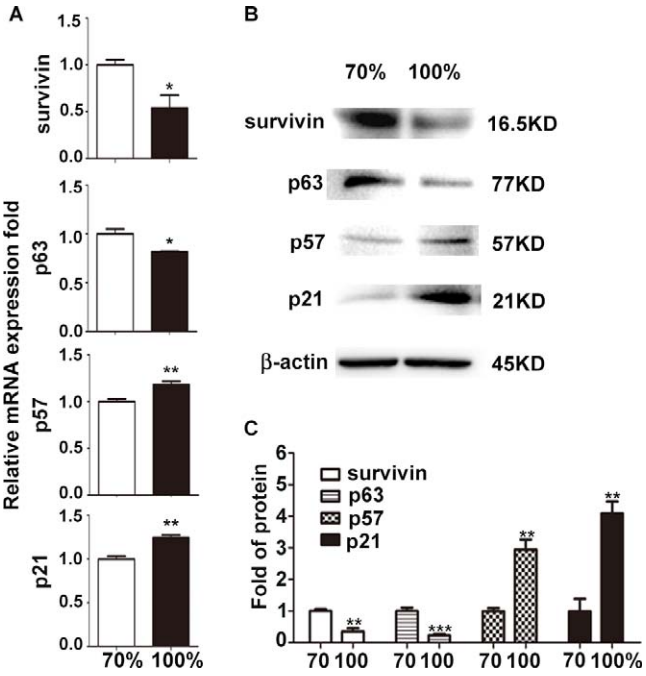

FIGURE 3. Survivin and related genes were differentially expressed in ex vivo–cultured PECs at different confluent stages. (**A**) Real-time PCR data of survivin, p63, p57, and p21 (relative fold) in primary cultured PECs at 70% and 100% confluence ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. 100% confluence). (**B**) Representative images of Western blotting of survivin, p63, p57, and p21. (**C**) Statistical analysis of Western blotting data. Data was represented as mean ± SEM, $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 100% confluence.

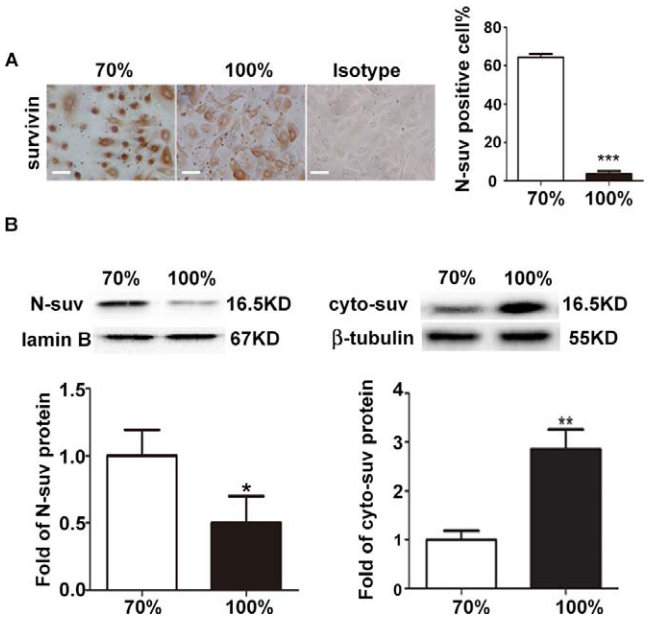

FIGURE 4. Cellular distribution of survivin in cultured PECs at different confluent stages. (**A**) Representative images and statistical analysis of immunohistochemical staining of nuclear survivin (N-suv) in PECs at 70% and 100% confluence ($n = 3$; $^{***}P < 0.001$ vs. 100% confluence). *Scale bars*: 50 μm. (**B**) Representative images and statistical analysis of Western blotting results of nuclear and cytoplamic survivin (N-suv and cyto-suv) in PECs at different confluent stages. Data were represented as mean ± SEM, $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. 100% confluence.

compared the expression of survivin in pterygium tissues with different clinical stages (Fig. 2B). Immunohistochemical staining showed that survivin was mainly expressed in the cytoplasm both in the head and in the body part of the pterygium tissues at quiescent stage. However, there were more nuclear survivin positive epithelial cells in the pterygium tissues at advanced stage, and this was prominent both in the head and in the body part of the pterygium tissues (Fig. 2B). Meanwhile, we detected the expression of phosphorylated survivin (P-survivin) in pterygium with immunofluorescent staining (Fig. 2B). We found that P-survivin was weakly expressed in the cytoplasm of the epithelial cells in pterygium at quiescent stage. In contrast, P-survivin expression was remarkably increased in the epithelium of pterygium at advanced stage. Moreover, there was obvious nuclear location of P-survivin in the advanced pterygium. We further performed p63 immunohistochemical staining on pterygium tissues. The results showed that p63-positive cells were sporadically distributed in the basal and suprabasal layers of epithelium in pterygium at quiescent stage. However, p63-positive cells were significantly increased in pterygium at advanced stage (Fig. 2B). These results suggested that there was correlation between pterygium staging and the differential expression pattern of survivin.

Survivin Promotes Proliferation of Pterygium Epithelial Cells

To further evaluate the role of survivin in pterygium, we performed ex vivo culture of PECs as described above. Real-time PCR results revealed that survivin mRNA level was higher in pterygium cells at 70% confluence than cells at 100% confluence ($P < 0.05$, $n = 3$; Fig. 3A). The mRNA level of p63 was higher ($P < 0.05$, $n = 3$) while cyclin-dependent kinase inhibitors p57 and p21, two proliferation inhibitors, were lower in 70% confluent PECs, compared with that of 100% confluent cells ($P < 0.01$, $n = 3$; Fig. 3A). Western blotting results confirmed that the protein expression of survivin and p63 was higher, while p57 and p21 was lower in PECs at 70% confluence, compared with that of 100% confluent PECs (Fig. 3B), and the difference was statistically significant (Fig. 3C).

To determine the cellular localization of survivin in pterygium epithelial cells, we performed immunohistochemical staining of survivin on PECs at 70% or 100% confluence. The results demonstrated that majority of the cells showed nuclear survivin expression when the cells were at 70% confluence (Fig. 4A, positive cells 64.43 ± 1.69%, $n = 3$), however, when the cells reached 100% confluence, nuclear staining of survivin dramatically decreased (positive cells 3.58 ± 1.48%, $n = 3$), while cytoplasmic expression increased. Western blotting on fractionation of cytoplasmic and nuclear protein revealed that 70% confluent cells showed more nuclear survivin than that in 100% confluent cells (2.01 ± 0.22 fold, $P < 0.05$, $n = 3$); however, cytoplasmic survivin was much higher in 100% confluent cells compared with that of 70% confluent cells (2.85 ± 0.41 fold, $P < 0.01$, $n = 3$; Fig. 4B).

We also performed immunofluorescent staining of P-survivin on PECs. The results showed that P-survivin was mainly expressed in the nuclear of PECs (Fig. 5A). There were approximately 60% P-survivin–positive cells (59.86 ± 4.12%, $n = 3$) when the PECs were at 70% confluence, while there were only approximately 8% P-survivin–positive cells (7.71 ± 0.29%, $n = 3$) when the PECs reached 100% confluence (Fig. 5A). Meanwhile, Western blotting confirmed that the amount of P-survivin in 70% confluent PECs

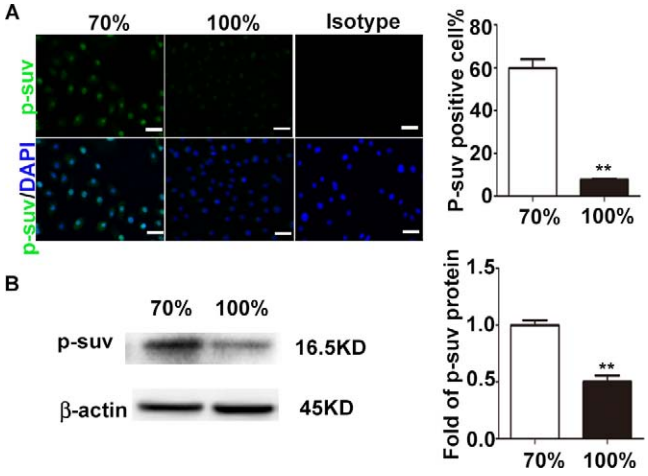

FIGURE 5. Phosphorylated survivin (P-suv) in PECs at different confluent stages. (**A**) Representative images of immunofluorescent staining of P-suv in PECs with 70% and 100% confluence. P-suv–positive cells were counted and correlated to total number of cells ($n = 3$; $^{**}P < 0.01$ vs. 100% confluence). *Scale bars*: 50 μm. (**B**) Representative images and statistical analysis of Western blotting of p-suv in PECs at different confluent stages. Data were represented as mean ± SEM, $n = 3$; $^{**}P < 0.01$ vs. 100% confluence.

was higher than that in 100% confluent PECs (Fig. 5B, $P < 0.01$, $n = 3$).

Survivin Gene Silence Inhibited Proliferation of Pterygium Epithelial Cells by Downregulation of P63 and Upregulation of p57 and p21

To further elucidate the mechanism of survivin on cell proliferation, RNA interference was performed to silence survivin gene by siRNA-survivin transfection in PECs. Immunostaining showed significant downregulation of survivin in PECs after siRNA application for 48 hours (Fig. 6A), the proliferation of PECs was also decreased after siRNA transfection (Fig. 6B, $P < 0.001$, $n = 6$), real-time PCR showed a successful knockdown of survivin gene after 48 hours (Fig. 6C), and its protein production was almost completely suppressed by siRNA-survivin, compared with siRNA-control, a noncoding negative control siRNA (Fig. 6D).

The gene expression of p63 was decreased in PECs after siRNA-survivin transfection. In contrast, p57 and p21 gene expression were upregulated after survivin knockdown (Fig. 6C). Western blotting results also confirmed the identical changes at protein level with statistical significance (Figs. 6D, 6E).

DISCUSSION

In the present study, for the first time, we found the differential expression pattern of survivin in pterygium at different clinical stages. In the quiescent stage, survivin was mainly expressed in the cytoplasm of pterygium epithelium. In contrast, survivin showed strong nuclear expression in the pterygium epithelium at advanced stage. In accordance with the expression pattern of survivin, we found that total p63, an epithelial cell proliferation potential marker, was expressed in low level in pterygium at quiescent stage, while was highly expressed at advanced stage. In other words, more epithelial cells in advanced pterygium may contain high proliferation capability, compared with quiescent pterygium. Based on previous

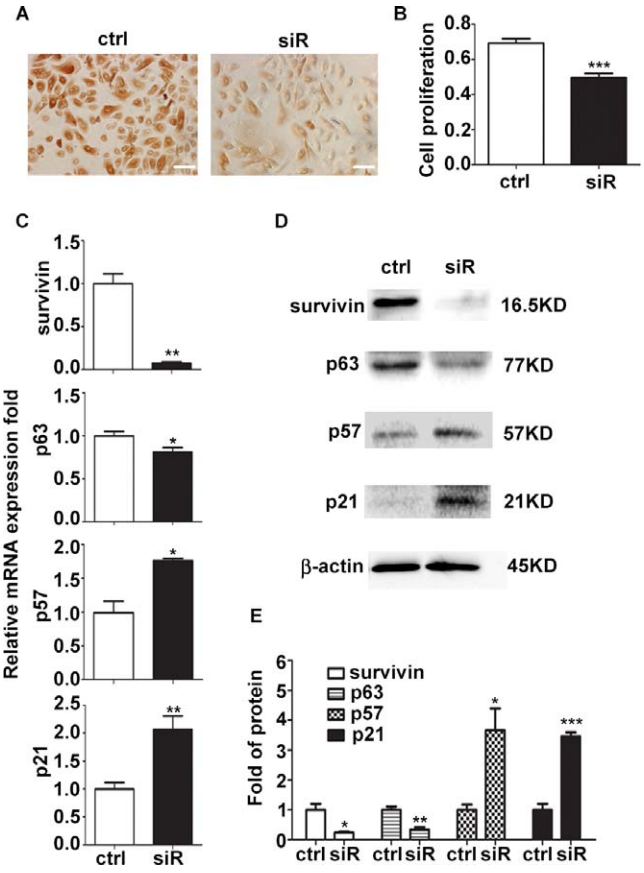

FIGURE 6. The effects of survivin silencing by siRNA interference on cell proliferation of PECs. (**A**) Representative images of immunohistochemical staining of survivin were shown after siRNA against survivin (siR) and siRNA noncoding negative control (ctrl) transfection. *Scale bars*: 100 μm. (**B**) Effect of siR and ctrl on cell proliferation of PECs by cell proliferation assay. Results are shown as mean ± SEM, $n = 6$; $^{***}P < 0.001$ versus control. (**C**) Real-time PCR data of survivin and related downstream genes (relative fold) in PECs after control and siR transfection ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ versus ctrl). (**D**) Representative images of Western blotting results of survivin and related downstream genes after siRNA-survivin interference. (**E**) Statistical analysis of survivin and related downstream genes. Data were represented as mean ± SEM, $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control.

reports that nuclear survivin promotes cell mitosis and proliferation,[43] while cytoplasmic survivin plays antiapoptotic function,[39] together with the p63 expression pattern, we proposed that survivin in advanced pterygium epithelium may play a role in promoting epithelial proliferation, which could induce progress of the disease. However, survivin in quiescent stage could play the potential role of antiapoptosis, which may contribute to the maintenance of the pterygium that would be studied in the future investigation.

Our study also showed higher expression of P-survivin in advanced pterygium than that in quiescent pterygium at the first time. As it was shown before, survivin phosphorylation played an important role in the chromosomal passenger complex, which can promote chromosome biorientation, targeting centromere in the cancer cell.[44] The phosphorylation of survivin on Thr34 mainly localized in the nucleus and played an important role during the malignant transformation.[45] In a way, cells could turn on lots of rescue mechanisms to activate and stabilize cytoprotection proteins for their abnormal growth during stress. For instance, survivin, stabilized by

phosphorylation, plays pivotal roles in cell survival and proliferation of trophoblastic cells upon stress.[46] The phosphorylation of survivin on Thr34 was also shown required to preserve cell viability at cell division.[47] In our study, we found P-survivin was mainly expressed in advanced pterygium rather than quiescent pterygium, indicating P-survivin may be the key player that could promote pterygium cell proliferation and prevent cells from apoptosis. Previous studies showed that some protein functional activation represents phosphorylation and nuclear translocation. The activation usually begins with the protein phosphorylation first, and then translocation from cytoplasm to nucleus, which further activates downstream signaling molecules for function (e.g., STAT3,[48] IRF,[49] β-catenin[50]). In this work, we found the phosphorylation and nuclear translocation of survivin in pterygium at the same time, and higher levels of these proteins were detected in both advanced pterygium and 70% confluent–cultured PECs. The most likely explanation for these observations is phosphorylation of survivin at Thr34 promotes its transport into nuclei of pterygium epithelial cells in some way which has not been elucidated and further research may be performed in the future study.

In order to further study the downstream signaling of survivin in pterygium epithelial cells, we applied pterygium epithelial cells cultured under different confluent stage to represent the different clinical stages of pterygium, since the cells with 70% confluence are mainly in exponential growth stage, which can mimic advanced stage with more proliferative cells, while the cells with 100% confluence will stop proliferation, thus can be considered as quiescent stage. The strong nuclear expression and phosphorylation of survivin in cultured PECs at 70% confluence further supported the notion that surviving, which presented in the nuclei of pterygium and was phosphorylated at advanced stage may play an important role in the hyperproliferation of the epithelium and progress of the disease.

The P63 gene encodes a member of the p53 family of transcription factors[51] and it expresses in limbal stem cells and transient amplifying cells which represent higher proliferation potential.[52] P57 (cycline-dependent kinase inhibitor 1C) is a strong inhibitor of cyclin/Cdk complexes and a negative regulator of cell proliferation. There was a previous report that p57 was regulated by Wnt signaling pathway in the process of human corneal epithelial stem cell proliferation.[37] P21 (cycline-dependent kinase inhibitor 1A) can inhibit the activity of cyclin-CDK2 or -CDK4 complexes, overexpression of survivin can result in release of p21 from Cdk4, and thus impacts cell cycle progression.[53] In the present study, survivin expression in cultured PECs at 70% confluence is associated with highly expression of p63 and low level expression of p57 and p21 (Fig. 3). A recent study also showed correlation of p63 and survivin in oral squamous cell carcinomas.[54] Survivin gene silence resulted in downregulation of p63 and upregulation of p57 and p21 (Fig. 6), suggesting that p63, p57, and p21 may be the downstream genes in survivin induced signaling pathway, and survivin may regulate pterygium epithelial cells through these genes.

Survivin has been considered as a potential molecular target in the treatment of cancer.[55,56] The first survivin inhibitor YM155 has been shown to suppress both survivin protein and mRNA expression,[57] and the phase I study was reported several years ago.[58] Other methods such as molecular antagonists, vaccination-based therapies, antisense oligonucleotides, and small hairpin RNA targeting survivin are also underway on investigation.[59–61] Future studies are obligatory to investigate such methods to prove that targeting survivin can affect progress of pterygium.

In conclusion, our study demonstrates that survivin gene is activated with phosphorylation and nuclear translocation in both pterygium tissues and primary cultured pterygium epithelial cells, survivin could be an important contributor to primary pterygium epithelial cell proliferation by regulation of p63-, p57-, and p21-mediated downstream signaling pathway. This may shed new light on the medication treatment of pterygium through regulation of survivin expression.

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